ZOOLOGIA
An International Journal for Zoology

ZOOLOGIA 38: e60598
ISSN 1984-4689 (online)
zoologia.pensoft.net

RESEARCH ARTICLE

A new species of aphid of the genus *Nipponaphis* (Hemiptera: Aphididae: Hormaphidinae) from China, inducing galls on the trunk of a witch-hazel (Hamamelidaceae)

Wei Wang[1,2], **Yongzhong Cui**[1], **Xiaoming Chen**[1,3], **Nawaz Haider Bashir**[1], **Hang Chen**[1,3]

[1] *Research Institute of Resources Insects, Chinese Academy of Forestry, Kunming, China*
[2] *NanJing Forestry University, Nanjing, China*
[3] *The Key Laboratory of Cultivating and Utilization of Resources Insects, State Forestry Administration, Kunming, China*
Corresponding authors: Hang Chen (stuchen6481@gmail.com), Xiaoming Chen (cafcxm@139.com)

http://zoobank.org/08C732DE-7C25-4143-9A1A-61EC2188CB6E

ABSTRACT. Plants and insects have co-existed for millions of years. Although research has been conducted on various insect species that induce galls on various plant tissues, information is particularly scarce when it comes to insects that form galls on the tough trunk of their host plants. This contribution describes the gall-inducing aphid *Nipponaphis hubeiensis* **sp. nov.** from the Zhushan County, Shiyan City, Hubei Province of China. This aphid induces enclosed galls with woody external layer on the trunk of *Sycopsis sinensis* (Saxifragales: Hamamelidaceae), an uncommon ecological niche in the aphid-plant interaction system. Morphological features for the identification of new species are provided. In addition, a partial sequence of the nuclear gene EF1α was amplified and sequenced to construct a cluster graph. Based on the clustering graph combined with morphology traits, the gall-forming aphid was classified into *Nipponaphis*. The unique ecological habits of this new aphid will bring innovative perspectives to the study of the evolution and diversity in aphid-host interaction.

KEY WORDS. Ecological niche, gall-inducing aphid, interaction, phylogeny, tannin content.

INTRODUCTION

Gall formation may have evolved as a mechanism to sequester galling insects, thus protecting other parts of the plant from potential damage (Price et al. 1987, Wool 2004). The aphid subfamily Hormaphidinae consists of three tribes: Nipponaphidini, Cerataphidini and Hormaphidini, and the members of each tribe are confined to a single plant genus *Distylium* (Hamamelidaceae), *Styrax* (Styracaceae) and *Hamamelis* (Hamamelidaceae), respectively (Aoki and Kurosu 2010). Galls of some Nipponaphidini have been found on *Sycopsis* and *Distyliopsis* (Hamamelidaceae) trees in Taiwan (Aoki and Kurosu 2010, Yeh and Ko 2017). These galls have different shapes and structure, for instance semi-spherical, spherical, spiny, tubular, bag-like, banana-bundle-shaped, or conical. They can be found on the petioles, leaves, twigs or veins (Chen and Qiao 2009). Some species of *Nipponaphis* (Nipponaphidini) form green, fig-shaped or globular galls (Aoki et al. 2015) and several species produce bottle-shaped galls, closed or open, with a single-cavity on the twigs of *Distylium chinense* (Chen et al. 2011, 2014).

There are 15 known species in *Nipponaphis* Pergande, 1906, distributed mainly in East Asia, Japan, India, Thailand, and China (Blackman and Eastop 1994). Some species of Nipponaphidine aphids are known to alternate between their primary hosts (genus *Distylium* spp., Hamamelidaceae) and secondary hosts (species of Moraceae, Lauraceae, and Fagaceae) (Blackman and Eastop 1994, 2006). Galls of species studied in detail (*Nipponaphis distyliicola* Monzen, 1934 and *Nipponaphis monzeni* Takahashi, 1958) take more than a year to mature, and the complete life cycle of the insect can last from two to five years (Monzen 1954). Takahashi (1962) reviewed the species on the host *Distylium* in Japan. Sorin (1987) illustrated differences in embryonic chaetotaxy, and Aoki et al. (2015) tabulated data on the chaetotaxy of first instar nymphs. Many Japanese species of *Distylium* have been reviewed and three have been transferred from the subgenus *Pseudonipponaphis* Ghosh and Raychaudhuri, 1973 to *Schizoneuraphis* van der Goot, 1917 (Ghosh 1973, Moritsu 1983). The species forming green galls on *Distylium* in Japan were reviewed using molecular and morphological data (Aoki et al. 2015). Two species, *Nipponaphis machili* (Takahashi, 1933) and *Nipponaphis minensis* Zhang & Guangxue, 1999, have been reported from China so far (Takahashi 1933, Zhang et al. 1999, Qiao et al. 2018). In Nipponaphidini, *Nipponaphis* exhibits a suite of plesiomorphic character states: body normal; antennae 3 or 4 segmented; apterae aleyrodiform; sturdy prosoma with mid and posterior setae; fused abdominal segments except VIII; abdominal tergum VIII with 4 setae; scattered sculptures on dorsum; cauda wart-like; siphunculus present and tarsi normal (Chen and Qiao 2012).

A new species, *Nipponaphis hubeiensis* sp. nov., forming woody-galls on the trunk of *Sycopsis sinensis* (Hamamelidaceae) is described. This increases the known number of species of *Nipponaphis* in China to three. This species occupies an unusual niche and is a promising model in studies of co-evolution between galling aphids and host plants.

MATERIAL AND METHODS

All samples were collected from Tianjiaba Town, Zhushan County, Shiyan City, Hubei Province, China, 32°11′48″N, 110°4′1″E, altitude 556 m, 15 August 2019, on *Sycopsis sinensis*. The collectors: Wei Wang, Yongzhong Cui and Kunfang Xiao. The voucher specimens and galls are deposited in the Insect Collection of Research Institute of Resources Insects, Chinese Academy of Forestry (RIRI-CAF). Morphological terms and abbreviations used in this paper generally follow Blackman and Eastop (2006) and Aoki et al. (2013).

The aphid samples were immersed in 75% ethanol, and then macerated for three minutes in 10% KOH solution before they were mounted on permanent slides. Adult specimens were cleared and mounted individually in Canada balsam on microscope slides according to the techniques described by Maw and Foottit (1998).

For electron microscopic observation, more than 20 aphid individuals with intact body were selected. They were placed on a conductive resin and gilded for 60 seconds in an ion plating machine (JS-1600, Beijing Htcy Technology Co., Ltd., China) and then observed under an electron microscope (TM3000, Hitachi High-Technologies Corporation, Japan).

For DNA extraction, 50 mg of aphids (about 200 individuals) were weighted and Ezupanimal genome DNA extraction kit (Sangon Biotech Co., Ltd., China) was used by following the manufacturer's protocol. The standard primer pair: EF2 (5′-ATGTGAGCAGTGTGGCAATCCAA-3′), EF3 (5′-GAACGTGAACGTGGTATCAC-3′) (Chen et al. 2014) were used to amplify EF1α gene from total genomic DNA.

PCR amplification was carried out in the Bio-RAD MyCycler thermal cycler using a volume of 50 μL: (Taq PCR master mixture 25 μL, ddH$_2$O 22 μL, DNA 1 μL, and primers (F/R) 2 μL). Amplification cycles were as follows: initial denaturation at 95 °C for 4 minutes; 35 amplification cycles involving denaturation at 94 °C for 1 minute, annealing at 56 °C for 1 minute and extension at 72 °C for 1 minute; final extension at 72 °C for 10 minutes. In order to ensure the accuracy of the sequence, the target fragment was incised and purified using a gel extraction kit (EZ-10 Column DNA Purification Kit) according to the manufacture instructions and purified PCR products were sequenced from both directions by Sangon Biotech Co., Ltd. Shanghai (China) sequencing instrument: ABI-3730XL gene sequencer.

Sequences of the elongation factor 1-alpha gene (EF1α) were used as molecular marker to investigate distances among Hormaphidinae species. The EF1α fragment of *Nipponaphis hubeiensis* sp. nov. was successfully amplified from genomic DNA. The forward and reverse sequences were aligned, trimmed off using Bioedit ver. 5.09 (Hall 1999) and assembled to a length of 1056 bp by DNAMAN ver. 9.0 (Woffelman 2004). The sequence was submitted to NCBI GenBank (http://www.ncbi.nlm.nih.gov) and got the GenBank accession number MT832325.

Additional EF1α sequences of 35 species from the tribes Nipponaphidini, Hormaphidini and Cerataphidini plus the Pemphigidae *Melaphis rhois* (Fordinae) as outgroup were downloaded from the GenBank database and used to construct the cluster graph. The accession numbers of the newly generated and downloaded sequences from the 36 taxa used in the analysis are given in Table 1. Multiple alignments of the sequences were carried out using ClustalW (Higgins and Sharp 1988), and then the results were verified manually. The distances were inferred using Neighbour-Joining (NJ) method and Kimura's two-parameter (K2P) model (Kimura 1980), undertaken with 1000 non-parametric bootstrap pseudoreplicates in MEGA Ver. 6.0 (Tamura et al. 2013). In addition, genetic distances among the samples are calculated (Table 2).

Tannin concentration in gall of *Sycopsis sinensis* was measured as followed by Scalbert (1992). For hydrolysable tannins, tannic acid was used as the standard and absorbance was measured at 276 nm.

TAXONOMY

Nipponaphis hubeiensis Wang & Chen, sp. nov.

Figs 1–7

http://zoobank.org/4BD49F44-ABC1-4C50-BF41-CE3E06C1C23E

Type material. Holotype: female, apterous viviparous with labeled China: Hubei, Shiyan city, Zhushan County, Tianjiaba Town on *S. sinensis*, 556 m, 32°11′48″N, 110°4′1″E, 15.VIII.2019, #20190021, coll. Wei Wang, Yongzhong Cui and Kunfang Xiao (RIRI-CAF). Paratypes: 14 females, same data as holotype except #20190022–35.

Diagnosis. Morphological characters which are supported the placement of new species in genus Nipponaphis by normal body, apterae aleyrodiform, antennae 4 segmented, sturdy prosoma with middle and posterior setae, fused abdominal segments except VIII, abdominal tergum VIII with 4 setae, scattered sculptures on dorsum, cauda, tarsi and presence of siphunculus. The new species is similar to other *Nipponaphis* in

Table 1. Collection information and GenBank accession numbers for the aphid samples analyzed in this study.

	Species	Host	Locality	GenBank accession number	References
1	*Nipponaphis hubeiensi* sp. nov.	*Sycopsis sinensis*	China: Hubei	MT832325	This study
2	*Nipponaphis distyliicola*	*Quercus glauca*	Japan: Shinkiba, Tokyo	AF454614	Huang et al. (2012)
3	*Nipponaphis monzeni*	*Distylium racemosum*	Japan: Shinkiba, Tokyo	AF454615	Huang et al. (2012)
4	*Nipponaphis coreana*	*Neolitsea sericea*	Korea	GU457844	Kim et al. (2011)
5	*Nipponaphis* sp.	*Machilus yunnanensis*	China: Yunnan	JX489711	Chen et al. (2014)
6	*Metanipponaphis lithocarpicola*	*Castanopsis sclerophylla*	China: Fujian	JX489705	Chen et al. (2014)
7	*Metanipponaphis* sp.	*Distylium chinense*	China: Hunan	JX489706	Chen et al. (2014)
8	*Neothoracaphis yanonis*	*Distylium chinense*	China: Hunan	JX489710	Chen et al. (2014)
9	*Neothoracaphis quercicola*	*Quercus acutissima*	China: Yunnan	JX489709	Chen et al. (2014)
10	*Neothoracaphis elongata*	*Quercus* sp.	China: Yunnan	JX489708	Chen et al. (2014)
11	*Parathoracaphis manipurensis*	*Castanopsis* sp.	China: Yunnan	JX489713	Chen et al. (2014)
12	*Parathoracaphis setigera*	Fagaceae	China: Yunnan	JX489714	Chen et al. (2014)
13	*Reticulaphis inflata*	*Ficus* sp.	China: Yunnan	JX489715	Chen et al. (2014)
14	*Reticulaphis* sp.	*Ficus* sp.	China: Yunnan	JX489716	Chen et al. (2014)
15	*Hamamelistes betulinus*	*Betula platyphylla*	Japan: Tokyo, Okutamako	AF454597	Huang et al. (2012)
16	*Hamamelistes kagamii*	*Betula grossa*	Japan: Yamanashi, Sanjonoyu	AF454601	Huang et al. (2012)
17	*Hamamelistes miyabei*	*Hamamelis japonica*	Japan: Yamanashi, Masutomi	AF454595	Huang et al. (2012)
18	*Hamamelistes spinosus*	*Betula nigra*	USA: Utah, Logan	AF454607	Huang et al. (2012)
19	*Hormaphis betulae*	*Betula ermanii*	China: Jilin, Changbai Mt	JX282846	Zhang et al. (2013)
20	*Hormaphis cornu*	*Hamamelis virginiana*	USA: Georgia, Athens	AF454612	Huang et al. (2012)
21	*Hormaphis hamamelidis*	*Hamamelis virginiana*	USA: Connecticut, Danielson	AF454613	Huang et al. (2012)
22	*Hormaphis similibetulae*	*Betula albosinensis*	China: Tibet, Linzhi	DQ493866	Huang et al. (2012)
23	*Ceratovacuna nekoashi*	Poaceae	China: Yunnan, Kunming	JX282827	Zhang et al. (2013)
24	*Ceratovacuna lanigera*	*Saccharum sinense*	China: Guangxi, Luocheng	JX282793	Zhang et al. (2013)
25	*Ceratovacuna graminum*	Poaceae	China: Zhejiang, Tianmu Mt	JX282842	Zhang et al. (2013)
26	*Ceratovacuna indica*	*Bambusa* sp.	India	JX282792	NCBI
27	*Pseudoregma alexanderi*	Bambusoideae	China: Fujian	JX489698	Chen et al. (2014)
28	*Pseudoregma koshuensis*	*Styrax suberifolia*	China: Guizhou, Daozhen	JX282779	Zhang et al. (2013)
29	*Pseudoregma bambucicola*	*Styrax suberifolia*	China: Zhejiang, Taishun	JX282782	Zhang et al. (2013)
30	*Pseudoregma sundanica*	Zingiberaceae	China: Hainan, Changjiang	JX282788	Zhang et al. (2013)
31	*Cerataphis bambusifoliae*	Unidentified bamboo	China: Fujian, Wuyi Mt	DQ493850	Huang et al. (2012)
32	*Cerataphis brasiliensis*	*Styrax benzoin*	Malaysia: Gombak	KX698154	NCBI
33	*Ceratoglyphina phragmitidisucta*	Poaceae	China: Fujian, Wuyi Mt	JX282850	Zhang et al. (2013)
34	*Astegopteryx formosana*	*Bambusa* sp.	China: Taiwan, Sun moon Lake	KX698155	NCBI
35	*Astegopteryx rhapidis*	*Cocos nucifera*	China: Hainan: Wenchang	JX282848	Zhang et al. (2013)
36	*Melaphis rhois*	*Rhus glabra*	USA: Arizona	KF601630	Ren et al. (2013)

Table 2. Estimates of evolutionary divergence between species of *Nipponaphis*.

	1	2	3	4	5	6	7
Nipponaphis hubeiensis sp. nov.	*						
Nipponaphis monzeni	0.0215	*					
Nipponaphis sp.	0.0437	0.0355	*				
Nipponaphis coreana	0.0465	0.0421	0.0347	*			
Nipponaphis distyliicola	0.0656	0.0549	0.0552	0.0543	*		
Metanipponaphis sp.	0.1007	0.0940	0.0953	0.0994	0.1016	*	
Metanipponaphis lithocarpicola	0.1419	0.1321	0.1278	0.1342	0.1404	0.1377	*

Based on EF1α gene fragment, K2P model used, only species clustered with Nipponaphis hubeiensis sp. nov. were selected.

having aleyrodiform present, prosoma, abdominal segments, head and thorax relatively longer than abdomen, wax gland plate lacking on dorsum, sculptures on dorsum present, number of setae on abdominal tergite VIII, frontal horns lacking and compound eyes 3-faceted. *Nipponaphis hubeiensis* sp. nov. may be separated from the closely related *N. monzeni* based on the following characters (those of *N. monzeni* between brackets): body oval, more than 1 mm long (body round, less than 1 mm long), antennae 4-segmented (antennae 3-segmented), cauda rectangular (cauda knobbed shape), setae present on antennal segment III (setae absent on antennal segment III) and submarginal setae on abdominal tergites II-VII absent (submarginal setae on abdominal tergites II-VII present). New species can be differentiated from *N. distyliicola* by four antennal segments, shape of sculptures on dorsum and shape of cauda. From the sympatric *N. machili* and *N. minensis*, the new species can be distinguished by antennae four segmented (antennae three segmented in *N. machili* and *N. minensis*), cauda rectangular (cauda knob-like in *N. machili* and *N. minensis*), head and pronotum completely fused (head separated from thorax in *N. machili* and *N. minensis*), primary rhinaria present on antennal segments III and IV (primary rhinaria present on antennal segment III in *N. machili* and *N. minensis*), granular protrusions on the dorsum (circular protrusions on the dorsum in *N. minensis*, and three dots like marking present on the mid of dorsum in *N. machili*).

Description. Body oval, head, thorax, antennae, mouthparts, legs and anal plate brown (Fig. 5); head and pronotum completely fused (Fig. 1); dense epandrium on the dorsum of body (Figs 1, 3). Body average length 1.25 mm (n = 20), width 0.62 mm; head 0.088 mm long, 0.07 times length of body; thorax 0.63 mm, 0.50 times length of body; and abdomen length 0.50 mm, accounting for 0.4 times length of body. Forehead curved, frontal tumor lacking, 5-6 setae on dorsum of head; compound eyes 3-faceted. Antennae short, 4-segmented, basal segment dilated (Fig. 6), total length 0.20 mm, 0.16 times of body length; 1-2 setae on segments I, II and III respectively, segment IV with 4 short setae; two round primary rhinaria distributed in the segment III and IV, respectively. Rostrum 5-segmented (Fig. 2), total length 0.26 mm, length of rostral segment IV and V 0.98 mm, 1.48 times longer than tarsus II of hind leg, 3 pairs short setae on segment I and II, segment III with 2 pairs of short setae, 4 terminal sensory setae on segment IV. Six pairs of setae on pronotum, 22 pairs of long and short marginal setae on lateral tergum, long setae on tergum 0.07-0.15 mm in length, 6.25 times of the diameter of antennal segment IV and 2.92 times of their short setae, slightly longer than setae on segment V. Two pairs of marginal setae on pronotum and mesonotum, abdominal tergites with 1 pair of marginal setae, no setae present on abdominal tergites I-VII, and 3-4 setae on VIII. A pair of conical siphunculi present on apex of abdomen (Fig. 7). Cauda rectangle in shape above anus (Fig. 4). Hind femur 0.12 mm long, 1.20 times longer than antennal segment IV; hind tibia 0.16 mm long, 0.11 times length of body; tarsus 3-fracted, hind tarsus II 0.07 mm long, tarsus terminal splits into two lateral claws, without paw pad.

Distribution. China (Hubei).

Etymology. The new species is named after the place where specimens were collected. The ‘hubeiensis’ is a toponymic referring to the type locality at Hubei province.

Primary host. *Sycopsis sinensis*.

Morphology and structure of galls

The morphology, structure and sites where galls grow are important biological characteristics of gall-inducing aphids, which have strong species specificity and are known as ‘extended phenotypic characteristics’ of aphids (Stern 1995, Stone and Schönrogge 2003, Wool 2004). Consequently, the morphology and characteristics of the galls described in this study can be used in species’ identification.

The gall forms on the trunk (Figs 8, 9), is oval, and has a closed single-chamber gall (Figs 10, 11), 30-50 mm in length and 25-35 mm in width. Fresh galls are pale-cyan, but turn brown after they mature. Some have protruding longitudinal veins (vascular bundles below), thick and woody gall chamber wall, hard and brittle texture. A cross section divides into two layers: the outer layer is thin, brown; the inner layer is thick, yellowish white (Fig. 10). Gall wall is about 3-4 mm thick and has many spindle-shaped pits on the inner surface of gall wall (Fig. 12). Wax is present inside the gall. At maturity, the galls form round holes as a secondary opening, while there are also round concave holes inside the un-burst galls (Fig. 11).

We speculated that the secondary opening is formed by the concave hole for aphids’ seasonal migration. This kind of gall is similar to Galla Chinensis, which is caused by the Chinese aphid *Schlechtendalia chinensis* (Bell, 1851), on the leaves of *Rhus chinensis* Mill. (Lee et al. 1997). We measure the tannin content of the gall produced by *N. hubeiensis* sp. nov. and compared it with the tannin produced by the Chinese horned gall (*S. chinensis*). We found that the tannin content in *N. hubeiensis* sp. nov. is extremely low in comparison, only about 3% (Fig. 13), which was obviously different from Chinese horned gall. Chen (1991) collected similar galls in Songbai Town, Shennongjia, Hubei Province, and found that the morphology of the ‘woody gallnut’ was similar to resembled Chinese horned gall form on *Rhus punjabensis* (Anacardiaceae) leaves and there is aphid species of *Kaburagia* Takagi, 1937 (Pemphigidae: Fordinae) in the region. The ‘woody gallnut’ might be a related to the aphid *Kaburagia ovogalIis* (Tsai and Tang 1945). For this reason, we carried out the molecular analysis to differentiate the species of aphids.

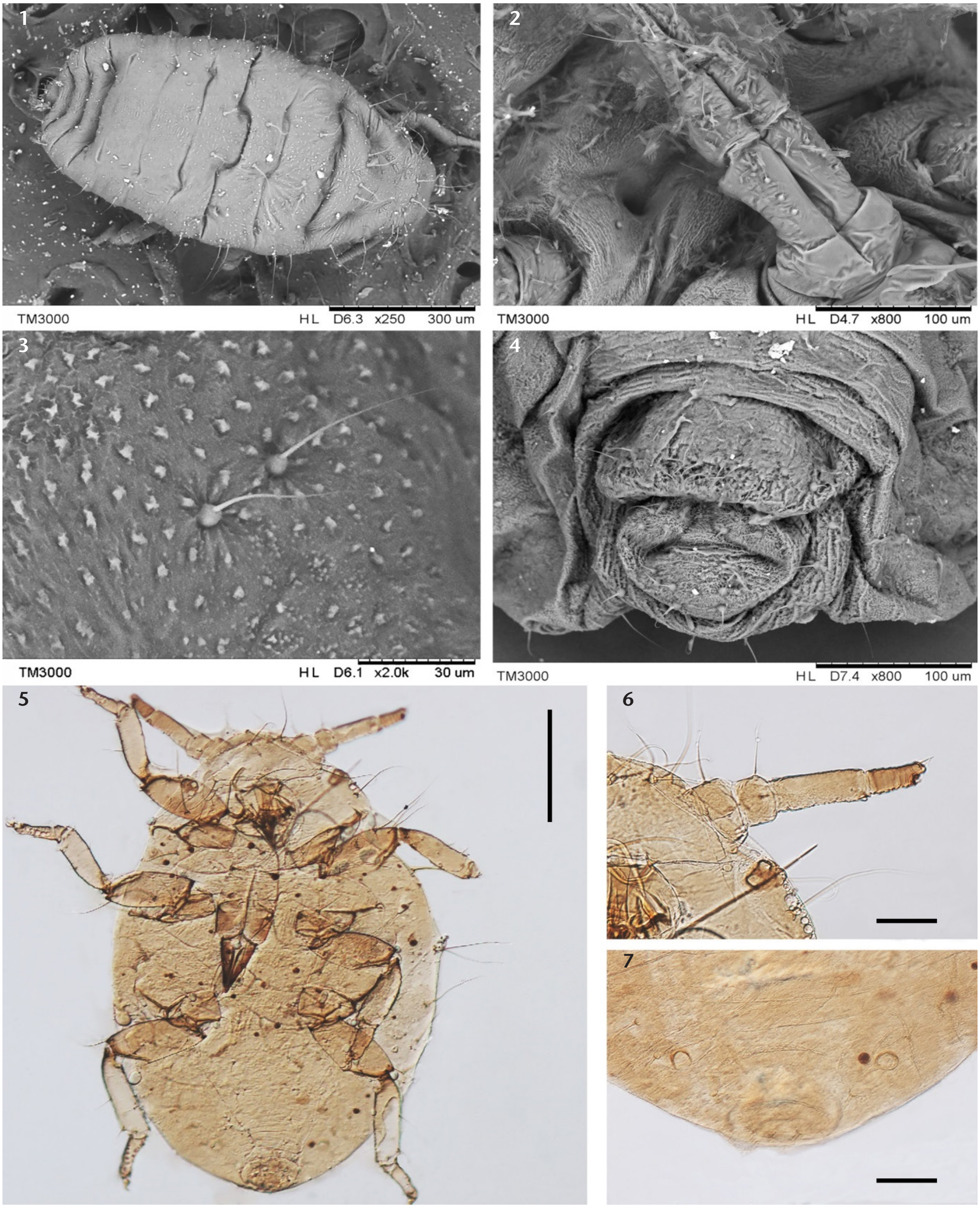
Molecular analysis

The topology distance-based clustering graph (NJ-tree) including 13 species of Nipponaphidini, 13 of Cerataphidini and 8 Hormaphidini resulted in robust branch support with the most bootstrap values higher than 70%. The tree is divided into three groups, consistent with the taxonomic classification (Fig. 14). *Nipponaphis hubeiensis* sp. nov. clustered in the genus *Nipponaphis*. *Nipponaphis monzeni* is the closest species to *N. hubeiensis* sp. nov. Interspecific K2P genetic distances also indicates the result with its maximum divergence 0.0656 (> 6%) from *N. distyliicola*, and minimum distance 0.0215 (> 2%) to *N. monzeni* (Table 2). Combined with the strong support from molecular evidence and morphological identification, the hypothesis that *N. hubeiensis* sp. nov. is not a close relative of Fordinae is confirmed and we are confident in erecting a new species for it in *Nipponaphis*.

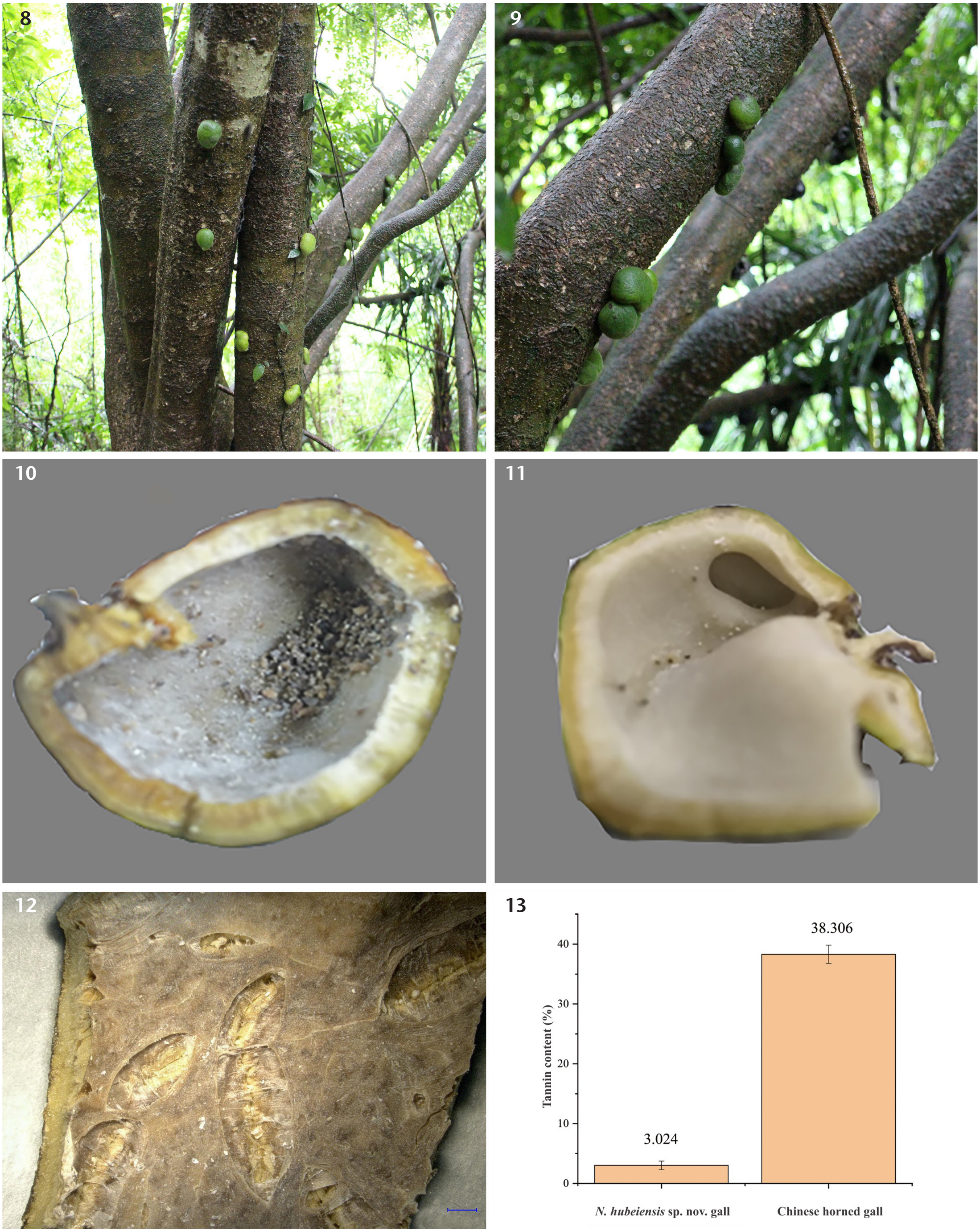
DISCUSSION

It has been questioned whether the interaction between gall-forming insects and their host plants is merely parasitic or whether it also benefits the host (Chen et al. 2020). Galls are special products of co-evolution between insects and host plants with unique ecological significance. The sites where galls grow are diverse, including leaves, veins, petioles, twigs, boughs and so on, but rarely will insects induce galls on the trunk of trees.

Many gall insects can manipulate the process of plant tissue differentiation and development, leading to gall production,



Figures 1–7. Micrograph of *Nipponaphis hubeiensis* sp. nov.: (1) dorsal view of body; (2) rostral segments; (3) tiny spinose projection and setae; (4) cauda; (5) body; (6) antennae and eyes; (7) siphunculus. Scale bars: 0.3 mm (1); 0.1 mm (2, 4, 6, 7); 0.03 mm (3); 0.5 mm (5).



Figures 8–13. (8, 9) Galls on the trunk; (10, 11) opened gall; (12) micrograph of gall inner layer (magnification ×200, scale bar – 1 mm); (13) tannin content of gall.

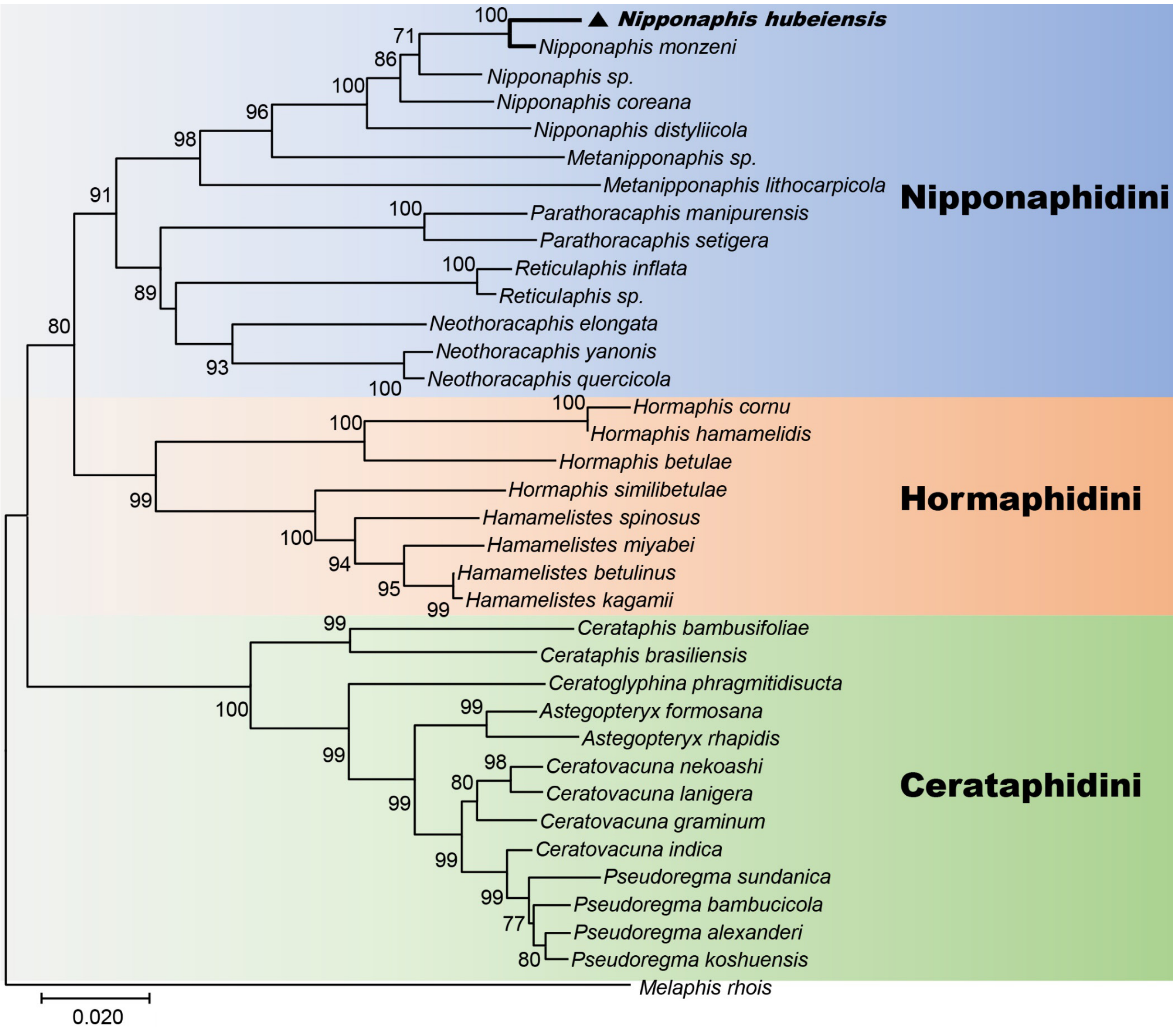


Figure 14. Neighbor-Joining tree for Hormaphidinae based on EF1α sequences. Bootstrap values higher than 70% are indicated on the nodes. The tree is rooted with *Melaphis rhois* (Pemphigidae: Fordinae). *Nipponaphis hubeiensis* sp. nov. clustered at the top with *Nipponaphis* species.

such as the formation of leaf galls, flower-like galls or fruit-like galls (Schultz et al. 2019). The sites where galls grow are dependent on the evolutionary strategy of the insect. The chemical components of galls, such as phenols, can effectively protect gall-causing insects from being parasitized or eaten (Rostás et al. 2013). However, the tannin content of the gall, calculated in this study at 3%, is not enough to protect the host plant itself. This gall, which develops on the trunk of the tree and forms a gall wall similar to the wood, has enhanced hardness and thickness. Compared with other types of galls, this one makes it difficult for the mouthparts of insects to pierce, thus providing effective defense. To some extent, the galls formed by *N. hubeiensis* sp. nov. are more vulnerable to damage by mammals because they are not located as high as those growing on branches and leaves. Galls not only provide a physical defense barrier, but also benefit from the chemical defense system built by the plants, which emit volatile substances that are able to repel mammals (Lee et al. 2015). Although we have not yet studied the chemicals emitted by the galls of the new species, we know that it is often accidental and rare that they are destroyed by mammals.

Various morphological structure and sites where galls grow reflect the adaptations of aphids to complex ecological environments. From the collected host plant, another kind of smaller cystic gall was found on the back of leaves. Forming galls on trunks certainly opens up a different niche with less competition for resources.

The discovery of *N. hubeiensis* sp. nov. is an interesting find for the study of ecological niches and towards a better understanding of the interaction and co-evolution between galling insects and their host plants.

ACKNOWLEDGEMENTS

We gratefully thank Kunfang Xiao (Hubei Natural Gallnuts Biotechnology Co., Ltd) for guiding to collect the samples of galls. This work was supported by the Fundamental Research Funds of CAF (CAFYBB2020QA003, CAFYBB2016MA006) and the Grant for Innovative Team of Yunnan Province (202005AE160011).

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Submitted: November 10, 2020
Accepted: March 17, 2021
Available online: May 11, 2021
Editorial responsibility: Ângelo Parise Pinto

Author Contributions: WW and YZC collected the specimens; WW described, illustrated the material and molecular analysis; WW, NHB and HC wrote and revised the manuscript; XMC and HC supervised the experiment.
Competing Interests: The authors have declared that no competing interests exist.